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CRYOPRESERVATION AND ITS APPLICATIONS – A BRIEF REVIEW

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ABSTRACT

Cryopreservation is a technique used to preserve biological material by cooling it to very low temperatures, typically below -130℃, in order to halt all biological activity. The storage and preservation of human tissues, cells, and organs is a very important process in clinical as well as in research. The technique called cryopreservation is commonly used for it. The cryopreservation involves the addition of cryopreservation agents (CPAs) also know as cryoprotectants while storing the tissues or organs in a sub-zero environment. The study of cryopreservation typically outlines the key aspects of the process, including the methods used, the rationale behind the study, the results obtained. If often includes information on the cryoprotectants used to prevent ice formation and cell damage, the cooling and thawing protocols, and the viability of the preserved material after thawing. Cryopreservation can be achieved by conventional freezing and vitrification methods. Due to complexity and intrinsic differences between isolated cells and tissues, evaluation of the success of cryopreservation for tissues and organs is quite different from that of cells and more difficult. The factors such as cooling rate, thawing rate, different CPAs, their effects, and alternative preservation methods and gives a systematic insight into how they affect the viability of biological sample.

KEYWORDS: Cryopreservation, Cryoprotectants, Vitrification, Thawing, Freeze-drying.

INTRODUCTION

Cryopreservation is the process which refers to the "preservation in the frozen state". It is derived from the Greek word 'KRYOS' meaning 'FROST'. The term cryopreservation means storage at low temperature such as in deep freezers (-80° C) in vapor phase nitrogen (-150° C) or in liquid nitrogen (-196° C). At such low temperatures, all the biological activities of the cells stop and the cell dies. Cryopreservation helps the cells to survive freezing and thawing. The ice formation inside the cells can break the cell membrane. This can be prevented by regulating the freezing rate and carefully choosing the freezing medium. Cryopreservation is the technique of cooling and preserving various biological samples such as storage of cells, tissues, and organs to maintain their viability. Biological materials such as cells, oocytes, spermatozoa, tissues, pre-implantation embryos, organs etc. are preserved in this technique and kept in extremely cold temperatures without affecting the cells viability. Dry ice and liquid nitrogen are also used in this method.[1]

History of cryopreservation

During 1950s James Lovelock suggested that increasing salt concentration in a cell as it dehydrated due to lose of water results in cell damage and he also proposed that the mechanism of action of cryoprotectant. In 1953, he suggested that damage to red blood cells during freezing was due to osmotic stress and that increasing the salt concentration in a dehydrating cell might damage it.[2]

In 1953, research work of Jerome k. Sherman led him to successfully freeze and thaw human sperm.

Cryopreservation was applied to human materials beginning in 1954 with three pregnancies resulting from the insemination of previously frozen sperm. Fowl sperm was cryopreserved in 1957 by a team of scientists in the UK directed by Christopher Polge. During 1963, Peter Mazur, at Oak Ridge National Laboratory in the U.S, demonstrated that lethal intracellular freezing could be avoided if cooling was slow enough to permit sufficient water to leave the cell during progressive freezing of the extracellular fluid. In 1983, Alan Trounson, was credited for successfully achieving a pregnancy after freezing early human embryos one to three days after fertilization.

PRINCIPLE OF CRYOPRESERVATION

The metabolic processes and biological divisions in the cells the principle of cryopreservation involves preserving biological material at very low temperatures, typically below -13 ℃, to prevent cellular damage and

biochemical reactions. When cells are frozen in aqueous suspension, often they are destroyed. In the 1940s Polge and others discovered the cryoprotective properties of glycerol. Then several chemicals, generically called cryoprotectant agents (CPAs), have been identified. This often done using cryoprotectants to minimize ice crystal formation and maintain cell viability during freezing and thawing processes. It is a process of preserving or storing cells, tissues, organs or any other biological materials from any potential damage by maintaining the materials at very low temperatures typically -80℃ using solid CO_2 or -196℃ using liquid nitrogen. In cryopreservation, very low temperatures are used to preserve living cells and tissues and maintain their viability. Unprotected freezing is normally lethal. Cryopreservation is based on the conversion of water present in the cells from a liquid to a solid state. Water constitutes approximately 80% of tissue mass, the freezing of water, both intra and extracellularly, imposes the largest over harmful biochemical, and structural changes that are thought to result in unprotected freezing injury. The cell water requires much lower temperature to freeze even up to -68 ℃ due to the presence of salts and organic molecules in the cells, in comparison to the freezing point of pure or tissues are almost stopped when stored at low temperature.[3]

Two independent theories exist that attempt to explain the harmful effects of freezing on cells:

1) Ice crystals mechanically disrupt cellular membranes thus making it impossible to obtain structurally intact cells after thawing
2) Increases in solute concentration occur to the remaining liquid phase as ice crystals form intra cellularly during cooling.

CRYOPROTECTANTS

Cryoprotectants are used to protect cell membrane integrity and intracellular environment to reduce or avoid the damage to the structure and properties of membrane lipids, proteins, and nuclei acids. They are divided into two categories depending on their cell membrane permeability.

1. Nonpermeable cryoprotectants: Sugars (e.g., trehalose and sucrose), Starches (e.g., hydroxyethyl starch), polyvinyl pyrrolidine, and polyethylene oxide, cannot enter cells and thus stay extracellular during cryopreservation. They are normally applied to protect cells during rapid cooling. After cooling starts, the extracellular solution will go through vitrification due to the extracellular presence of cryoprotectants. As a result, the water flux between inside and outside of cells will be inhibited as the viscosity 0f extracellular increases. When a slow freezing rate is applied, there is sufficient time for intracellular water to move out of cells under the osmotic pressure, resulting in a reduction in cell volume.

2. Permeable cryoprotectants: That can enter cells, including dimethyl sulfoxide (DMSO), glycerol, ethylene, glycol, and propylene glycol, can be used to induce the vitrification of intracellular environment before ice crystal formation, thus preventing excessive loss of cell volume.[4]

More cryoprotectants have been developed from natural products. A novel soybean flour natural products made up of protein (34.20g/100g), carbohydrate (36.00g/100g), and lipid (21.10g/100g) has been demonstrated to prevent Bacillus subtilis SB-MYP-1 cell damage after freeze drying stress. Rhodiola sachalinensis saccharide extracted from the rhizome of herba rhodiolae has also been used as a new cryoprotectant for bovine spermatozoa. It has been reported that a natural zwitterionic molecule, 1-carnitine, could greatly improve the survival rate of ornamental cichlid fish found after exposure to a cold shock. Antifreeze proteins found in marine organisms have also been utilized in cryopreservation of cells, embryos, and organs.

MECHANISM

The mechanism of action of CAPs is complex and is not fully understood. According to the commonly accepted theory of colligative action, CAPs increase solute concentration both within the cell and extracellularly, thereby suppressing ice formation. For this purpose, so called penetrating (or intracellular) CAPs [e.g., dimethyl sulfoxide (DMSO), glycerol, propanol, and methanol] must be able to cross the cell membrane readily and penetrate the cell without significant toxicity. There also is a group of nonpenetrating (or extracellular) CAPs (e.g., sucrose and trehalose) whose mechanism of action is thought to be related at least in part to their stabilizing interaction with cell membranes. This property also may explain the cryoprotective activities of certain large molecular weight compounds such as hydroxyethyl starch and polyvinyl propylene. Theoretical models of cryoprotection typically evoke the colligative theory, but full explanation of CAPs action is yet to be established.[5]

Most commonly used cryoprotectants

Cryoprotectants are crucial in storing biological samples at a deep cryogenic temperature. Cryoprotecting agents lower the melting point of water when dissolved in it and prevent ice formation by increasing the total concentration of all the solutes present in the sample and hence protect the cells. Cryopreserved samples need to have the same level of viability upon the recovery, cryoprotectants must penetrate into the cells and should have lower toxicity. This article discusses the most commonly used cryoprotectants used in life science research.[6]

1. Dimethyl sulfoxide (DMSO)

The Russian scientist Alexander Zaytsevun synthesized DMSO in 1866. DMSO is the most commonly used cryoprotectant to protect cells. It is a cell penetrating agent and protectants biological samples from intracellular ice formation. It represents a cost-effective option with minor toxicity. DMSO can cause DNA

methylation which in turn altered cells and histones thereby becoming DMSOs.

2. Ethylene glycol

60% ethylene glycol is incapable of forming crystals when mixed with 40% water which makes it one of the ideal cryoprotectants. Ethylene glycol interferes with the hydrogen bonds in water molecules to bind. This prevents the formation of ice crystals and lowers the waters freezing point. It is also toxic to some level causing gastrointestinal irritation, using inflammation, and edema.

3. Glycerol

Glycerol is a colorless and odorless viscous liquid. It is a good kosmotropic agent and forms stable hydrogen bonds with water molecules. It contributes to the stability and structure of water-water interactions. This characteristic makes it difficult to form ice crystals.

4. Propylene Glycol

Propane-1, 2-diol a non-irritating colorless, odorless, synthetic organic compound. It is miscible with water, chloroform, and acetone. It has the property of an autonomous anti-freeze.

5. Polymers

Non-diffusible synthetic polymers like polyvinyl alcohol, polyethylene glycol (PEG), and Hydroxyethyl starch are some selective cryoprotective agents used for biological samples. They have a good potential to decrease the size of ice crystals.

TYPES OF CRYOPRESERVATION

According to different cell types, the cryopreservation processes can roughly be grouped into four slow freezing types:[7]

1. Slow freezing

The most appropriate storage method of small volumes cryopreservation of several microencapsulated cells. It must be carefully selected that the CPA solution, sample preparation, cooling, thawing, and CPA removal.

2. Vitrification (solidification of the aqueous part of the cell or tissue into a non-crystalline glassy phase)

There are three primary constituents in the cryoprotectant involve polyvinyl pyrrolidine (PVP), Dimethyl Sulfoxide (DMSO), and Glycerol. It must be used the cryoprotectant concentrations which sufficient to prevent the crystallization of ice altogether. High concentrations of cryoprotectants are possibly harmful to cells. The lowered concentration can be realized through using very rapid cooling, and even more rapid warming. Add the HEPES into the cryopreservation to buffering the pH of the cryopreservation, it also has the effect of making the cryopreservation an isotonic solution of mitochondria.

3. Subzero nonfreezing storage

It uses a refrigerating device that generates a variable magnetic field. Even if without the use of cryoprotectants, the sample would be cooled to a supercooled state. It was verified the method has the ability to suppressing anaerobic metabolism and decrease harm in the hearts of large animals.

4. Preservation in the dry storage

The ultimate goal is fully realized when stable long-term storage without the use of liquid nitrogen and bulky freezers, it becomes available. It is prove that increase cell survival at low water contents by loading cells with the protective disaccharide trehalose. It needs a low level of residual water is required to achieve viability after rehydration.

STEPS OF CRYOPRESERVATION

The cryopreservation of plant cell culture followed by the regeneration of plants involves the following steps:

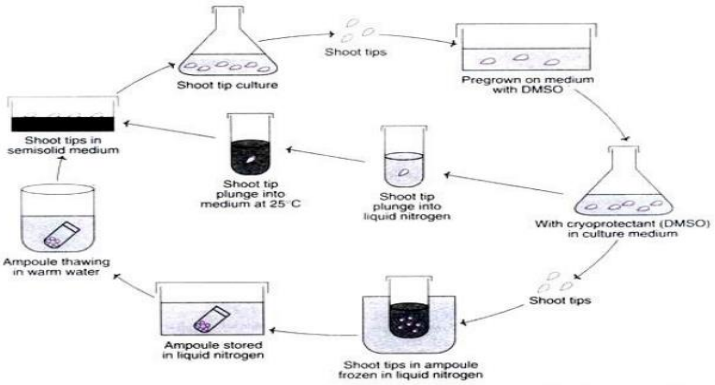
Selection of material

Material chosen for cryopreservation should be far as possible in meristematic state.

For selecting a material, a number of facts are taken into account:

1. The nature of cells.
2. The density of cells in the vials to be preserved.

Cell cultures are generally preserved in lag or early exponential phase of growth. Young, highly cytoplasmic and small cells which are no vacuolated and in small aggregates are good materials to be selected for cryopreservation. In some species, it may be important to use highly embryonic cell cultures, non-embryonic or poorly embryogenic show poor or no regrowth after thawing.[8]



"Fig. 1"

1. Development of sterile tissue culture

The selection of plant species and the tissues with particular reference to the morphological and physiological characters largely influence the ability of the explant to survive in cryopreservation. Any tissue from a plant can be used for cryopreservation e.g., meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Among these, meristematic cells and suspension cell cultures, in the large phase or log phase are most suitable.

Addition of cryoprotectants and pretreatment

Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. The freezing point and super-cooling point of water are reduced by the presence of cryoprotectants. As a result, ice crystal formation is retarded during the process of cryopreservation.

The several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose mannose, glucose, proline, and acetamide. Among these, DMSO, sucrose and glycerol are most commonly used. A mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cell or tissues.

Freezing

The sensitivity of the cells to low temperature is variable and largely depends on the plant species.

Four different types of freezing methods are used:

1. Slow-freezing method

The tissue or the requisite plant material is slowly frozen at a slow cooling rate of 0.5-5°C/min from 0°C to -100°C, and then transferred to liquid nitrogen. The advantage of slow-freezing method is that some amount of water flows from the cells to the outside. This promotes extracellular ice formation rather than intracellular freezing. As a result of this, the plant cells are partially dehydrated and survive better. The slow-freezing procedure is successfully used for the cryopreservation of suspension culture.

2. Rapid freezing method

This technique is quite simple and involves plunging of the vial containing plant material into liquid nitrogen. During rapid freezing, a decrease in temperature -300° to -1000°C/min occurs. The freezing process is carried out so quickly that small ice crystals are formed within the cells. Further, the growth of intracellular ice crystals is also minimal. Rapid freezing technique is used for the cryopreservation of shoot tips and somatic embryos.

3. Stepwise freezing method

This is a combination of slow and rapid freezing procedures, and is carried out in a stepwise manner. The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes and then rapidly cooled by plunging it into liquid nitrogen. Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.

4. Dry freezing method

Some workers have reported that the non-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic injuries. In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

Storage

Maintenance of the frozen cultures at the specific temperatures is as important as freezing. In general, the frozen cells/tissues are kept for storage at temperatures in the range of -70 to -196°C. However, with temperatures above -130°C, ice crystals growth may occur inside the cells which reduces viability of cells. Storage is ideally done in liquid nitrogen refrigerator at 150°C in the vapour phase, or at -196°C in the liquid.

The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability. For long term storage, temperature at -196°C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential. It

is necessary to check the viability of the germplasm periodically in some samples. Proper documentation of the germplasm storage has to be done.[9]

The documented information must be comprehensive with the following particulars

Taxonomic classification of the material
History of culture
Morphogenic potential
Genetic manipulation done
Soma clonal variations
Culture media
Growth kinetics

Thawing

Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water temperature 37-45℃ bath with vigorous swirling. By this rapid thawing occurs (at the rate of 500-750℃), and this protects the cells from the damaging effects ice crystals formation. As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water at temperature 20-25℃. This transfer is necessary since the cells get damaged if left for long in warm (37-45℃) water bath. The cryopreserved material (cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thawing becomes less critical.

Re-culture

The thawed germplasm is washed several times to remove cryoprotectants. This material is then re-cultured in a fresh medium following standard procedure. Some workers prefer to directly culture the thawed material without washing. This is because certain vital substances, released from the cells during freezing, are believed to promote in vitro cultures.

Measurement of survival/viability

The viability/survival of the frozen cells can be measured at any stage of cryopreservation or their thawing or re-culture. The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures. Staining techniques using triphenyl tetrazolium chloride (TTC), Evans blue and fluorescein diacetate (FDA) are commonly used. The best indicator to measure the viability of cryopreserved cells in their entry into cell division and regrowth in culture. They can be evaluated by the following expression.

Plant regeneration

The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant. For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed and grown. Addition of certain growth promoting substances besides maintenance of appropriate environmental conditions is often necessary for successful plant regeneration.[10]

A selected list of plants in various forms that have been successfully used for cryopreservation is given.

A selected list of plants is various forms that are successfully cryopreserved
Table 1

Plant material	Plant species
Cell suspensions	Oryza sativa
	Glycine max
	Zea mays
	Nicotiana tabacum
	Capsicum annum
Callus	Oryza sativa
	Capsicum annum
	Saccharum sp
Protoplast	Zea mays
	Nicotiana tabacum
Meristems	Solanum tuberosum
	Cicer arietinum
Zygotic embroys	Zea mays
	Hordeum vulgare
	Manihot esculenta
Somatic embryos	Citrus sinensis
	Daucus carota
	Coffea arabica
	Nicotiana tabacum
	Citrus sp
	Atropa belladonna

APPLICATIONS OF CRYOPRESERVATION

1. Assisted reproductive technologies (ART)

Cryopreservation is extensively used in ATR to preserve embryos, sperm, and eggs. This allows individuals undergoing fertility treatments to store their reproductive cells for future use, increasing the chances of successful conception.

2. Tissue and organ transplantation

Cryopreservation enables the long-term storage of tissues and organs, extending the viability of donor tissues and organs for transplantation. This is particularly crucial for organs like heart kidneys, and livers, where timely availability is critical for saving lives.

3. Biomedical research

Cryopreservation is essential in preserving cells, tissues, and organs for biomedical research purposes. It enables researchers to maintain cell cultures, tissue samples, and organoids for studying disease mechanisms, drug development and regenerative medicine.

4. Conservation of biodiversity

Cryopreservation plays a vital role in conserving biodiversity by preserving gametes, embryos, and tissues samples from endangered species. This helps in maintaining genetic diversity and preventing the extinction of vulnerable species.

5. Preservation of genetic resources

Agricultural and livestock industries utilize cryopreservation to conserve genetic resources of valuable breeds and strains. This ensures the availability of superior genetic material for breeding programs improving livestock productivity and disease resistance.

6. Medical and clinical applications

Cryopreservation is employed in storing stem cells, blood products, and immune cells for medical treatments and therapies. It also facilitates the preservation of tissues grafts, such as skin grafts for burn victims, and bone grafts for orthopedic surgeries.

7. Drug development and toxicology

Cryopreserved cells and tissues serve as valuable models for drug screening, toxicity testing, and preclinical studies. They allow researchers to assess the efficacy and safety of pharmaceutical compounds in a controlled environment, reducing the need for animal testing.

8. Food preservation

Cryopreservation techniques are used in preserving food products, such as fruits, vegetables, and seafood. By freezing food at ultra-low temperatures microbial growth is inhibited, extending the shelf life of perishable items while maintaining their nutritional quality.

Cryopreservation has a wide range of applications spanning from healthcare and biotechnology to conservation and food preservation. Its versatility and effectiveness in preserving biological materials make it indispensable in various fields of science and industry.[11]

ADVANTAGES AND DISADVANTAGES

Table-2

Advantages	Disadvantages
1. Preservation of tissues/organs: Cryopreservation allows for long term storage of tissues, organs, or even embryos which ca be used for transplantation, research, fertility preservation.	**1. Cell damage:** The process of freezing and thawing can cause damage to cells and tissues, affecting their viability and functionality.
2. Extended viability: it can extend the viability of biological materials, preserving them in a state of suspended animation until needed.	**Technical challenges:** cryopreservation requires specialized equipment and expertise, making it costly and inaccessible in some settings.
3. Ethical concerns: There are ethical considerations surrounding the use of cryopreserved embryos and genetic material, particularly regarding their disposal or use without consent.	**3. Ethical concerns:** There are ethical considerations surrounding the use of cryopreserved embryos and genetic material, particularly regarding their disposal or use without consent.
4. Fertility preservation: Cryopreservation enables individuals facing medical treatments that may affect fertility, such as chemotherapy, to preserve their reproductive cells for future use.	**4. Limited success rates:** While cryopreservation techniques have improved, there are still limitations, particularly with certain cell types or tissues, leading to lower success rates in some cases.
5. Species preservation: It has the potential to contribute to the preservation of endangered species by storing genetic material for future reproductive purposes.	**Long-term storage risks:** There is a risk of equipment failure or power outage leading to loss of stored samples, emphasizing the need for reliable storage facilities and backup systems.

CONCLUSION

Cryopreservation holds promise for preserving tissues, organs, and even whole organisms by freezing them at ultra-low temperatures. While its widely used for preserving cells and reproductive materials, its application to more complex structures like organs is still being researched. Ethical logistical, and technical challenges remain, but ongoing advancement suggest a

hopeful future for cryopreservation in medicine and beyond.[12]

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